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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Breast cancer is the most prevalent of all cancers and it is the second cause of cancer death among women. Centrosome defects have been implicated in cancer formation and we showed that they can be detected very early on in this process, in the precancerous lesions. The centrosome protein, pericentrin, is overexpressed in many types of tumors including breast, and in this study we tried to understand the role of pericentrin in carcinogenesis. We found that pericentrin down regulation by RNA interference causes cytokinesis defects which can contribute to aneuploidy and thus cancer formation. We also identified a centrosome "damage" G0/G1 checkpoint that is dependent upon intact p53 and p38 signaling. Moreover, we showed that pericentrin down regulation is involved in the differentiation process through inhibition of ciliogenesis. Uncontrollable cell division in cells that should otherwise be in a differentiated state is the very first step in cancer formation. Better understanding of all of these processes is necessary for implementing a better treatment for breast cancer in order to reduce the mortality rates associated with this deadly disease.

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Introduction

Genetic instability and aneuploidy are hallmark of most cancers including breast tumors and they correlate with advanced stages of the disease [1, 2]. Aneuploidy is defined as state of an alter chromosome number, while its partner chromosome instability (CIN) is the rate of change [3]. The molecular players involved in this process are unknown, however, chromosome missegregation during mitosis and/or cytokinesis failures are the most likely candidates that could cause genetic instability. Pericentrin overexpression in tissue culture cells was shown to cause defects in mitotic spindles and cytokinesis [4]. Moreover, pericentrin is overexpressed in many malignant tumors [5, 6], and centrosomes of these tumors are abnormal in number, shape and size. Cell lines with ectopic expression of pericentrin show anchorage-independent growth in soft agar. Therefore, a better understanding of the precise role of pericentrin in mitosis would shed light on its role in tumor formation.

Recently, centrosomes have been elevated to much higher level in terms of their importance in cell cycle progression. They are no longer just a microtubule organizing centers, but they have been implicated in cytokinesis, G1/G0 checkpoint, cell cycle progression and differentiation [7-11]. One can easily imagine how centrosome over replication during mitosis could contribute to multipolar spindle formation and missegregation of chromosomes. The daughter cell of such division could survive by inheriting extra copies of chromosomes containing genes that would give the cell a growth advantage, or by losing tumor suppressor genes. Failure in cytokinesis would be another route to formation of polyploidy which could offset genetic homeostasis and pave the road for carcinogeneis.

Body

The training I have received in the Doxsey lab has been excellent. I have learned many new and interesting techniques including small interfering RNAs (siRNA), ectopic gene expression, immunofluorescence assays and live imaging of cells, just to name a few. We had many successful collaborations with the clinicians in the cancer field including Dr. Dario Alteri (our new Cancer Center Director) and Dr. German Pihan (an oncologist and long-time collaborator) to continually bridge the gap between basic and clinical research.

The University of Massachusetts Medical School has been an outstanding training facility for young scientists offering diverse and expert technical assistance available on site. The seminar series are also very well organized and the invited speakers are always the leaders in the field ranging from tumor biology to cell motility and cytoskeleton. I have attended many seminars that have gaven me a better understanding of cancer biology, cell division, signaling and cell motility. I have also directly participated in a discossion series to present the most recent papers in the field and discuss the data in a collegial setting.

I also plan to attend the 6th International Workshop on Chromosome Segregation and Aneuploidy at Cortona, Italy in September 2004. I will present a poster examining the role of pericentrin in cell cycle progression and ciliogenesis. Participation in this meeting will provide me with valuable insight and knowledge of the most recent advances in the field of chromosome segregation and aneuploidy. Many leaders in the field have been selected to present seminars including Jonathon Pines, Michel Bornens, Stephen Doxsey, Erich Nigg, Ed Salmon and Conly Rieder.

Unexpected difficulties

We have proposed in the first Aim to use breast cancer biopsies from early lesions in order to see if we can predict the aggressive disease. We did find centrosome defects associated with the early precancerous lesions [12], however, after 911 we have more difficulties obtaining the necessary samples and this aim is slowly progressing. Despite those difficulties we will continue to work on the proposed Aims.

Deviation from the original statement of work

The goal of this project is to understand the relationship between pericentrin and breast cancer progression and I have not deviate from this course. Aims 2 and 3 were to analye the effect of pericentrin overexpression in breast cancer cell lines and its cooperativeness with other oncogenes in carcinogenesis. However, with the recent development of siRNA, downregulation of the protein seemed to be a better approach to this problem due to high specificity of siRNA technology. We know that pericentrin overexpression has a disruptive effect on microtubules (Fig. 1) that could make it difficult to accurately interpret the results, while siRNA does not seem to have an effect on the microtubule network [13, 14]. Therefore, we used siRNA to study the role of pericentrin in cell cycle progression in normal cells. We will next extend this approach to breast cancer cell lines at different stages of carcinogenesis. Otherwise, we will continue the course laid out in my statement of work for Aims 1-3.

Key Research Accomplishment

We found that downregulation of pericentrin by siRNA causes defects in (1) final stages of cytokinesis, (2) cell cycle progression, G1/G0 arrest and (3) inhibits ciliogenesis.

(1) Pericentrin silencing produces the exact cytokinesis phenotype that we showed previously for the centriolar protein centriolin [11]. The dividing cells are able to go through cytokinesis. However in the final stages of cytokinesis they remain connected by long strands of cytoplasm and fail to cleave. The cells that failed to cleave formed multinucleated cells [11]. Since, both centriolin and pericentrin had similar phenotype, we tested for biochemical interactions using immunoprecipitation assays. Both endogenous co-immunoprecipitated in normal retinal epithelial cell line (RPE1) (Fig. 2). Moreover, we showed that they were codependent on each other for their centrosome localization. When centriolin was reduced by siRNA both centriolin and pericentrin were specifically mislocalized from the centrosome and vice versa (Fig 3 a and b). It would be interesting to see if pericentrin and centriolin will show codependency in their midbody localization, where one may predict they play the most important role during cytokinesis. Future studies will be directed towards understanding the fate of the cells that failed cytokinesis and more indepth study of the final stages of cytokinesis in order to identify all of the molecular players.

(2) The cytokinesis defects were observed very early after pericentrin siRNA (about 14 hr) in normal, diploid RPE1 cells with an intact p53 pathway. Similar phenotypes were also observed in cells with a compromised p53 pathway, such as Hela, after few days of siRNA, typically 72 hr. We were not able to observe mitosis in the RPE1 cells after several days of siRNA treatment since the cells arrested in the G0/G1 stage [11]. We went on to discover that this arrest is dependent upon intact p53 (Fig. 4). Our results indicate a "centrosome checkpoint" operating in G0/G1 monitoring an intact fully functional centrosome. This checkpoint could function as a guard against genomic instability by preventing cells with compromised centrosomes from entering the cell cycle.

(3) While looking for the best marker of G0/G1 cells to identify more precisely the type of arrest that we were seeing in the pericentrin knockdown cells, we examined the formation of primary cilia as a marker for G0 cells. The primary cilium is a tiny organelle present on most differentiated cells of human body that serves as flowmeter and whose function is largely unknown. To our surprise we noticed that primary cilia did not form when pericentrin levels were decreased by siRNA, even when we stimulated the cells to produce cilia by serum starvation [10]. Recently, primary cilia loss was correlated with polycystic kidney disease and intraflagellar transport (IFT) proteins were shown to be responsible for cilia assembly [15]. Polycystin 2 is another protein that localizes to cilia and it is implicated in polycystic kidney disease [16, 17]. Based on our loss of cilia in pericentrin knockdowns, we looked to see if pericentrin cooperates with IFT proteins and polycystin 2 in ciliogenesis. Using lysates of RPE cells, endogenous IFT proteins and polycystin 2 were precipitated with pericentrin. Moreover, we showed that pericentrin is present in the purified IFT fractions and that IFTs are present in centrosome fractions. Our immunofluorescence data showed that IFT proteins and polycystin 2 colocalize at the basal body and are codependent on each other for this localization [10]. Therefore, we conclude that pericentrin, IFT and polycystin 2 form a complex in human cells and they are necessary for cilia formation. It would be interesting to see if cilia formation is also abrogated in breast cancer cells and to see if it correlates with the disease. In future studies we will use mammary epithelial cells to see if we can abrogate ciliogenesis in those cells. Moreover, we will look to see if cilia are defective in breast cancer cells as they are in kidney cysts.

Reportable Outcomes

• Minisymposia presentation 2003, "siRNA-mediated centrosome damage activates a G1 checkpoint", K. Mikule, A. Jurczyk, A. Gromley, S.J. Doxsey, 43rd annual meeting of the ASCB, San Francisco, CA.

• Poster presentation 2004, "Pericentrin forms a complex with intraflagellar transport proteins and polycystin-2 and is required for primary cilia assembly", A. Jurczyk, A. Gromley, S. Redick, J. San Agustin, G. Witman, G. Pazour, and S. Doxsey, 6th International workshop on Chromosome Segregation and Aneuploidy, Cortona, Italy.

• Manuscript 2003, "A novel human protein of the maternal centriole is required for the final stages of cytokinesis and entry into S phase", A. Gromley, A. Jurczyk, J. Sillibourne, E. Halilovic, M. Mogensen, I., Groisman, M. Blomberg, and S. Doxsey, Journal of Cell Biology, 161:535-545 (A. Gromley and A. Jurczyk contributed equally to this work).

• Manuscript 2004, "Pericentrin forms a complex with intraflagellar transport proteins and polycystin-2 and is required for primary cilia assembly", A. Jurczyk, A. Gromley, S. Redick, J. San Agustin, G. Witman, G. Pazour, D. Peters, and S. Doxsey, Journal of Cell Biology, in press.

• Manuscript 2004, "Pericentrin forms a complex with centriolin and is required for the final stages of cytokinesis and entry into S phase" A. Jurczyk, A. Gromley, and S. Doxsey, Current Biology, in preparation.

• Manuscript 2004, "Centrosome insult activates a p53-dependent G1 checkpoint", Keith Mikule, A. Jurczyk, A. Gromley, and S. Doxsey, in preparation.

• Cell lines have been established for imaging living cells for centrosome behavior and chromosome segregation including RPE1-GFP-histone2B, RPE1-GFP-centrin2, RPE1-GFP-EB1, and RPE1-GFP-αtubulin, human mammary epithelial cell line - HME1-GFP-histone2B (more of the breast lines are currently being made).

Conclusions:

This year has been very productive. Novel and significant discoveries were made with the identification of "centrosome integrity checkpoint" and linking centrosomes with the differentiation process of ciliogenesis. These findings could impact significantly the understanding of the cell cycle and differentiation and the role they play in tumorigenesis. Moreover, the G1 checkpoint and ciliogenesis could provide new targets for cancer treatments.

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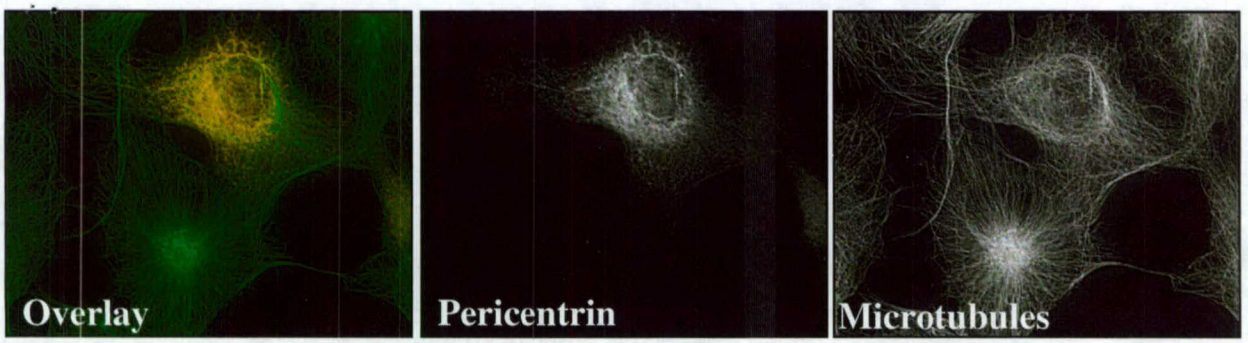

Fig.1

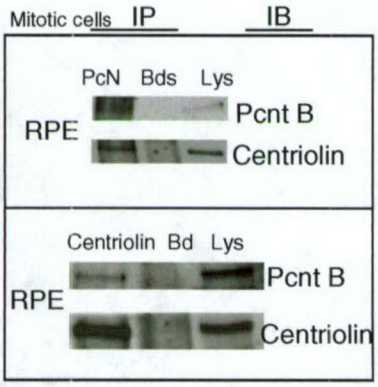

Fig. 2

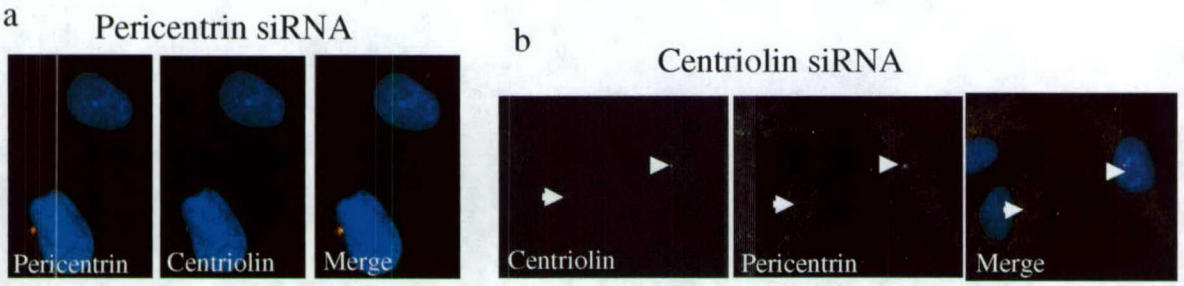

Fig. 3

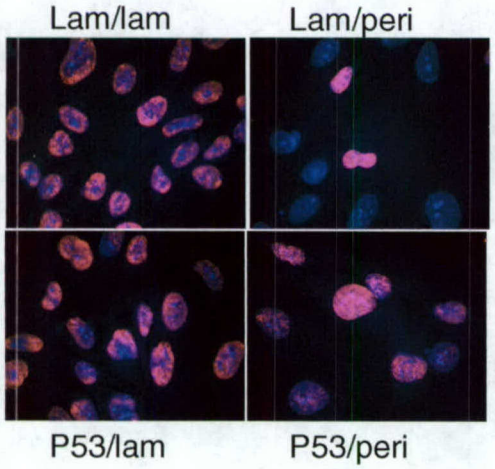

Fig. 4

Legends

Figure 1. Overexpression of HApericentrin disrupts microtubules organization in interphase cells. Cells were fixed in methanor and stained with antibodies to pericentrin and αtubulin and processed for indirect immunofluorescence.

Figure 2. Western blot of (top) pericentrin immunoprecipitation showing that both centriolin and pericentrin were pulled down with the immunoprecipitation, (bottom) centriolin immunoprecipitation. Beads (Bd) were used as controls. Lysates (Lys) are indicated. Mitotic RPE1 cells were used for both immunoprecipitations.

Figure 3. Pericentrin and centriolin are codependent on each oter for their centrosome localization. (a) pericentrin siRNA mislocalizes centriolin from the centrosome, (b) centriolin siRNA mislocalizes perisentrin from the centrosome in RPE1 cells, arrows point to the centrosome.

Figure 4. Brdu analysis indicates cells are arrested in G1 and that arrest is p53 dependent. Arrested cells fail to incorporate brdu. Following the knockdown of indicated gene, cells were grown in the presence of Brdu for 24 hours. 80% of arrested cells failed to incorporate Brdu. Note that cells knocked down for p53 failed to arrest following pericentrin knockdown.